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Enzyme-Coupled Assay for β-Xylosidase Hydrolysis of Natural Substrates

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We describe here a new enzyme-coupled assay for the quantitation of D-xylose using readily available enzymes that allows kinetic evaluation of hemicellulolytic enzymes using natural xylooligosaccharide substrates. Hydrogen peroxide is generated as an intermediary analyte, which allows flexibility in the choice of the chromophore or fluorophore used as the final reporter. Thus, we present D-xylose quantitation results for solution-phase assays performed with both the fluorescent reporter resorufin, generated from *N*-acetyl-3,7-dihydroxyphenoxazine (Amplex Red), and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), whose corresponding radical cation has an absorbance maximum at ~400 nm. We also describe a useful solid-phase variation of the assay performed with the peroxidase substrate 3,3′-diaminobenzidine tetrahydrochloride, which produces an insoluble brown precipitate. In addition, kinetic parameters for hydrolysis of the natural substrates xylobiose and xylotriose were obtained using this assay for a glycosyl hydrolase family 39 β-xylosidase from *Thermoanaerobacterium* sp. strain JW/SL YS485 (Swiss-Prot accession no. O30360). At higher xylobiose substrate concentrations the enzyme showed an increase in the rate indicative of transglycosylation, while for xylotriose marked substrate inhibition was observed. At lower xylobiose concentrations k_{cat} was 2.7 ± 0.4 s^{-1}, K_m was 3.3 ± 0.7 mM, and k_{cat}/K_m was 0.82 ± 0.21 $mM^{-1} \cdot s^{-1}$. Nonlinear curve fitting to a substrate inhibition model showed that for xylotriose K_i was 1.7 ± 0.1 mM, k_{cat} was 2.0 ± 0.1 s^{-1}, K_m was 0.144 ± 0.011 mM, and k_{cat}/K_m was 14 ± 1.3 $mM^{-1} \cdot s^{-1}$.

There is much current interest in enzymes that degrade starch, cellulose, and hemicellulose due to their increasing use in biobleaching and for the conversion of biomass to chemical feedstocks and fuels (3, 12, 13). Our group has been pursuing gene discovery and improvement of enzymes that degrade biomass through the use of directed molecular evolution (7, 17–20), in which a library of mutated enzymes is screened to select for clones with improved performance under the screening assay conditions employed (10, 14). One enzyme family of interest is the β-xylosidases (EC 3.2.1.37). These enzymes are exo-type glycosidases that remove xylose monomers from the nonreducing end of xylooligosaccharides and serve as one component of a multienzyme milieu that biodegrades hemicellulose (13). Thus far, β-xylosidase activity assays have relied mainly on the use of substrate analogs consisting of xylose O-linked to a chromophore or fluorophore, such as nitrophenyl or β-umbelliferyl. While the substrate analogs mentioned above are extremely useful for gene discovery and protein structure-function studies (2, 9, 16), use of these compounds in directed evolution efforts could be misleading. Thus, one could select from an enzyme library variants with improved activity for substrate analog hydrolysis that for xylooligosaccharide glycosidic bond hydrolysis are either inactive or not improved. The converse is also possible, so that enzymes in the library with high activity for hydrolysis of the native glycosidic bond are either inactive or not improved for hydrolysis of the substrate analogs. Thus, for improved selection an in vitro screening procedure that emulates the anticipated final commercial-scale bioreactor process conditions is desired, and it requires a selection assay that allows monitoring of depolymerization of native xylan or xylooligosaccharide substrates to xylose monosaccharides. Various chemical methods to quantitate monosaccharide formation based on the reducing capacity of the sugars are not useful since the natural substrates themselves contain a reducing group and are usually present in large excess. We describe here a new enzyme-coupled xylose assay and an accompanying method to characterize a *Thermoanaerobacterium* sp. strain JW/SL YS485 glycoside hydrolase family 39 β-xylosidase (XylB; Swiss-Prot accession no. O30360) (9). In addition, this assay appears to be amenable to conversion to both solution and solid-phase high-throughput formats for use in gene discovery and directed evolution studies, and it is potentially useful as a reporter activity assay for characterization of other hemicellulose-degrading enzymes, such as xylanases.

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MATERIALS AND METHODS

Plasmid DNA of p-Xylo 1.1 was isolated using a Qiaprep spin miniprep kit (QIAGEN). The plasmid DNA was digested using EcoRI (New England Biolabs [NEB], Beverly, MA) and electrophoresed using a 1.5% low-melting-point agarose gel (Cambrex Biosciences, NJ), and the desired band containing *xylB* was excised and purified using Zymoclean gel DNA recovery (Zymo Research, Orange, CA). PCR primers were designed to subclone the gene into a pET-22b(+) expression vector with a C-terminal His_6 tag (Novagen, San Diego, CA). The sequence of the forward primer was 5′-ATG ATT AAA GTG ATA GTG CCA GAT TTT TCC-3′, and the sequence of the reverse primer was 5′ GCG CTC GAG ATA TCC GTT TAT CTT GCT ATC ATC AAG TCC T-3′.

The reverse primer generated a XhoI site that was used for directional cloning

into the vector. The cloning strategy consisted of a 5′ blunt-end fragment and a 3′ XhoI cohesive end. PCR amplification was performed by traditional methods using *Pfu* Ultra Hotstart DNA polymerase (Stratagene, La Jolla, CA) and 0.5 μM of each primer. PCR generated a 1.5-kb fragment with a 5′ blunt end and a 3′ XhoI restriction site. The reaction product was purified using DNA clean and concentrator-25 (Zymo Research, Orange, CA). A XhoI site was generated by restriction enzyme digestion, and the fragment was cleaned up using a Min Elute gel extraction kit (QIAGEN). The vector was prepared for cloning by restriction digestion with NdeI (NEB, Beverly, MA). The desired band was purified using a Min Elute gel extraction kit (QIAGEN), and the 5′ overhang was filled using T4 DNA polymerase (Promega, Madison, WI). After this, the pET-22b(+) vector was purified and cut with XhoI. The cleaned vector was dephosphorylated using calf intestine phosphatase (NEB, Beverly, MA). Ligation was performed using T4 ligase (NEB, Beverly, Madison) and a 5:1 insert/vector ratio. Chemical transformation was done using JM109 (Promega, Madison, Wisconsin) at 16°C overnight. Positive clones were confirmed by colony PCR of plasmid preps (QIAGEN) using the primers mentioned above.

The expression vector was used to transform chemically competent *Escherichia coli* BL21(DE3) cells, and transformants were selected by overnight growth at 37°C on Luria-Bertani agar plates amended with 75 μg/ml carbenicillin (LB_{carb}). Overexpression was achieved by incubating a single colony overnight at 37°C in 10 ml LB_{carb} with 0.5% glucose, from which 8 ml was used to inoculate 250 ml LB_{carb}. The culture was allowed to grow to an optical density of 2 to 3 at 30°C, and then isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM) was added and growth was allowed to occur for an additional 16 h at 30°C. Then 50-ml aliquots were pelleted, and each pellet was lysed at room temperature for 20 min by adding 3 ml of a Bug-Buster solution (Novagen 70584) containing 1 μl/ml Benzonase (Novagen 712053), 1 μl/ml r-Lysozyme (Novagen 71114), 1 μl/ml protease inhibitor cocktail set III (Calbiochem 539134), and 5 mM β-mercaptoethanol. The supernatant solution was adjusted to contain 300 mM NaCl, 10 mM imidazole, and 50 mM phosphate buffer (pH 8.0) before binding to Ni-nitrilotriacetic acid resin (QIAGEN 1018142) at 4°C. The resin was transferred to a small column (Bio-Rad 7311550) and washed with 50 mM phosphate buffer (pH 8.0) containing 1 mM β-mercaptoethanol, 1 μl/ml protease inhibitor cocktail, 300 mM NaCl, and 10 mM imidazole. The protein was eluted using the same buffer except that the imidazole concentration was increased to 250 mM. Fractions containing the enzyme were buffer exchanged using NAP-5 desalting columns (Amersham 17085301) into 50 mM phosphate (pH 6.0), 10% glycerol, and 200 μM phenylmethylsulfonyl fluoride and stored at −80°C. Protein concentrations were obtained using Coomassie Plus reagent (Pierce 1856210) by following the manufacturer's protocol. Enzyme fractions were analyzed using polyacrylamide gel electrophoresis by following the manufacturer's protocol (Invitrogen, Carlsbad, CA), and the final protein purity was estimated to be 73% from gel densitometry using an AlphaImager imaging system (Alpha Innotech Corp., San Leandro, CA).

Enzymatic assay. The solution-phase assay for β-xylosidase natural substrate hydrolysis activity is performed in two steps. The first step is an endpoint assay of the β-xylosidase enzyme being evaluated, in which the glycosidic bonds of a soluble xylooligosaccharide substrate (xylobiose or xylotriose in this study; Wako Chemicals USA, Inc., Richmond, VA) are hydrolyzed by the enzyme, and D-xylose is generated. The β-xylosidase enzyme evaluated here is a family 39 retaining glycoside hydrolase cloned with a C-terminal His_6 tag from a *Thermoanaerobacterium* sp. genomic library (Swiss-Prot accession no. O30360) (9). In the second step, the amount of xylose liberated is quantified using an enzyme-coupled kinetic assay in which pyranose oxidase (pyranose:oxygen 2-oxidoreductase) converts the D-xylose hydrolysis product to 2-keto-D-xylose with concomitant formation of H_2O_2 (equation 1). In a subsequent (more rapid) reaction catalyzed by horseradish peroxidase, the hydrogen peroxide generated oxidizes the chromogen 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) (Sigma A-9941) to form the colored ABTS radical cation (equation 2), and the rate of formation is monitored at 420 nm.

$$\text{D-xylose} + O_2 \xrightarrow{\text{pyranose oxidase}} \text{2-keto-D-xylose} + H_2O_2 \quad (1)$$

$$H_2O_2 + 2\text{ABTS(H)} \xrightarrow{\text{peroxidase}} 2H_2O + 2\text{ABTS}^{\cdot +} \quad (2)$$

For liquid-phase assays we also employed *N*-acetyl-3,7-dihydroxyphenoxazine (Amplex Red; Molecular Probes, Eugene, OR) in place of ABTS, which reacts with a 1:1 stoichiometry with H_2O_2 in a reaction catalyzed by peroxidase to produce highly fluorescent resorufin, which was detected by excitation at 550 nm and emission at 590 nm (22).

Time course studies were performed initially to determine the linear range of the assay. It was established that the rate of substrate hydrolysis using either xylobiose or xylotriose at a concentration of 4 mM was in each case linear ($R^2 > 0.99$) for at least 90 min at 45°C. Generally, 16 different substrate concentrations were used to assess the kinetic parameters, and the amount of enzyme was chosen so that the proportion of substrate hydrolyzed ranged from 1 to 3% when xylobiose was used at various concentrations as the substrate and from <1% to 7% when xylotriose was used at various concentrations as the substrate. The judicious use of enzyme concentration to limit substrate conversion, coupled with the previously established linearity of enzyme activity with respect to time at the assay temperature, served to clearly established that there was linearity of the hydrolysis rate for the substrate concentrations and reaction times used to assess the kinetic parameters. In a typical solution-phase assay 105 μl of 50 mM phosphate (pH 6.0) containing 0.1% bovine serum albumin (BSA) and the β-xylosidase enzyme (2.7 nM for the xylotriose assays and 18 nM for the xylobiose assays) was placed into PCR strip tubes and chilled to 0 to 4°C. The reaction was initiated by adding 20 μl of an appropriately diluted xylobiose or xylotriose solution, gentle mixing, and incubation at 45°C for 45 min using a PCR machine (MJ Research, Watertown, MA) preequilibrated at 45°C. At the end of the incubation period, the temperature was programmed to immediately ramp up to 100°C for 7 min to quench the reaction, followed by cooling to 0°C. This completed the β-xylosidase endpoint assay, and the samples were then stored in a freezer or immediately analyzed using the xylose enzyme-coupled quantitation assay. When the reaction being studied was catalyzed by a His-tagged enzyme and the enzyme was not readily heat inactivated, it was also possible to quench the reaction by freezing on dry ice and to prevent further substrate hydrolysis by adsorption of the enzyme at 0 to 4°C onto Ni-nitrilotriacetic acid resin (data not shown). No-incubation controls were included, in which the enzyme was heat inactivated in the presence of the various substrate concentrations, followed by incubation in parallel with the assays. This compensated for small D-xylose impurities in the xylobiose and xylotriose substrates, as well as for any substrate hydrolysis that may have occurred either before the 45°C incubation temperature was reached or during the ramp-up of temperature involved in the heat inactivation quenching step. After brief centrifugation, the contents of the PCR strip tubes were transferred to Millipore Multi-Screen 96-well filtration plates (Millipore MAHVN4510) and centrifuged to obtain a filtrate free of precipitated protein, which would have interfered with the subsequent spectrophotometric assay. Aliquots (100 μl) of the filtrate were transferred to standard flat-bottom 96-well plates (Greiner Bio-One 655101) along with 100 μl of a reaction buffer consisting of 125 mM phosphate (pH 7.4), 400 μM ABTS or 400 μM Amplex Red, 1.7 U/ml peroxidase (Sigma P6782), and 1 U/ml pyranose oxidase (Sigma P4234). The plates were gently mixed for 30 s, and the rates were obtained at 25°C using a Spectramax M2 plate reader (Molecular Devices Corporation, Sunnyvale, CA) from the initial slopes (200 to 800 s; $R^2 > 0.99$) of the change in absorbance at 420 nm for ABTS or the change in fluorescence (excitation at 550 nm, emission at 590 nm) for Amplex Red. The D-xylose concentration in the assays was calculated using a D-xylose standard curve generated on the same microtiter plate.

To compare the kinetic parameters obtained with the native substrates with those obtained using chromophore-tagged xylose substrate analogs, kinetic spectrophotometric assays were also performed using the substrates *o*- and *p*-nitrophenyl-β-D-xylopyranoside at 35°C in 50 mM phosphate (pH 6.0) containing 0.1% BSA and measurement of the change in absorbance at 400 nm. Rates of hydrolysis were obtained from the initial slopes measured from 60 to 400 s ($R^2 > 0.99$). The rate of nitrophenyl-β-D-xylopyranoside hydrolysis was quantified using standard curves generated with either the *ortho*- or *para*-nitrophenyl analog.

A solid-phase variation of the assay was also developed, in which the chromogen 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Aldrich 261890), which in the presence of H_2O_2 in a peroxidase-catalyzed reaction produces an insoluble brown precipitate, is used. To test the solid-phase assay, *E. coli* BL21(DE3)(pLysS) (Novagen, Madison, WI) was transformed either with empty pET-22b(+) vector as a negative control (vector only, Fig. 1) or with the pET-22b(+) vector containing the β-xylosidase gene insert as a positive control (β-xyl, Fig. 1) and grown overnight in LB_{carb}. The cultures were then aliquoted into 96-well microtiter plates and robotically gridded (Q-Bot; Genetix USA Inc., Boston, MA) onto a Nytran SPC membrane (Schleicher & Schuell Bioscience, Keene, NH). The membrane was then placed onto LB_{carb} growth and induction agar containing 1 mM IPTG and incubated overnight at 30°C. The membrane was then carefully lifted from the growth and induction agar and placed face down onto a combined lysis and indicating agar containing 50 mM phosphate,

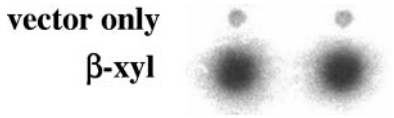


FIG. 1. Solid-phase assay of *E. coli* BL21(DE3) transformed with vector only or with the β-xylosidase gene (β-xyl). Bacterial colonies were grown and expressed in the presence of 1 mM IPTG at 30°C overnight on a Nytran SPC membrane placed on LB_{carb}. Cell lysis and detection were performed as described in the text by incubating the membrane overnight at room temperature using DAB as the peroxidase substrate. The image was obtained using an AlphaImager imaging system (Alpha Innotech Corp., San Leandro, CA).

pH 7, 1.5% (wt/vol) low-melting-point agar, 0.86 mg/ml mixed xylooligosaccharides (approximately 1 mM xylobiose and 1 mM xylotriose; Wako Chemicals USA, Inc., Richmond, VA), 1 U/ml peroxidase, 0.5 U/ml pyranose oxidase, 1 mM DAB, 1 mg/ml hen egg white lysozyme (Sigma L7651), 1 mg/ml polymyxin B sulfate (Sigma P1004), and 1 μl/ml protease inhibitor cocktail III (Calbiochem 539134). The lysis and indicating agar was prepared by dissolving the agar in the phosphate buffer and allowing it to cool to less than 40°C before the remainder of the components were added. Lysis and hydrolysis of the mixed xylooligosaccharides were allowed to occur overnight at room temperature, and the membrane was analyzed using an AlphaImager imaging system (Alpha Innotech Corp., San Leandro, CA).

RESULTS

The standard curve generated using the enzyme-coupled xylose assay and ABTS as the chromophore was linear up to the highest concentration of xylose tested (320 mM), and the limit of detection was in the low μM range. The assay worked equally well using Amplex Red, as shown in Fig. 2 for concentrations up to 30 μM for Amplex Red and 60 μM for ABTS. The limit of detection of the Amplex Red fluorescence-based assay was on the order of 1 μM or less, and we were able to generate a linear standard curve from 0.1 μM to 1.0 μM xylose with a correlation coefficient (R^2) of >0.99 (data not shown). However, for use in the β-xylosidase assay (see below) the sensitivity achieved using ABTS was more than sufficient since the concentration of xylose released in the assays was in the range from 3 to 330 μM. In order to determine the suitability

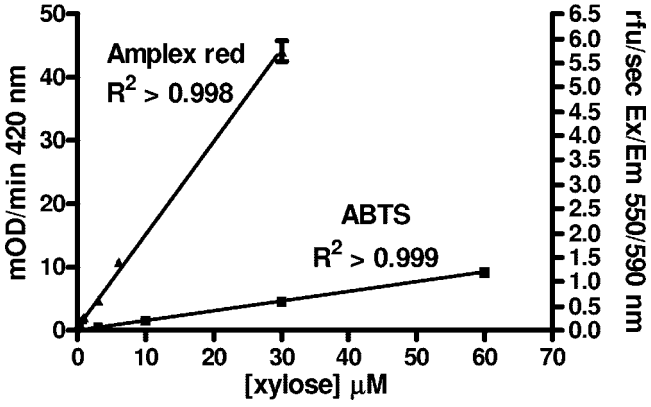


FIG. 2. D-Xylose standard curves generated using ABTS as the chromophore or Amplex Red as the fluorophore. The error bars are generally about the size of the symbols. mOD/min 420 nm, 10^{-3} unit of optical density at 420 nm/minute; rfu/sec Ex/Em 550/590 nm, relative fluorescence units/second at an excitation wavelength of 550 nm and an emission wavelength of 590 nm.

of the assay for detecting xylose in the presence of a large xylooligosaccharide substrate concentration, the background signal was measured and found to be 2.5% and 1.5% for xylobiose and xylotriose, respectively. The observed background may have been due to the presence of trace levels of xylose in the xylooligosaccharide substrates, as revealed by thin-layer chromatography (data not shown). Also, it was found that the xylose standard curve generated in the presence of 12 mM xylobiose was identical to that generated in the absence of xylobiose, while the xylose standard curve generated in the presence of 12 mM xylotriose showed at most a 2% reduction in sensitivity.

The kinetic data obtained for the nitrophenyl-β-D-xylopyranosides and xylobiose at high substrate concentrations all deviated from Michaelis-Menten kinetics, presumably due to transglycosylation. Thus, increases in both k_{cat} and K_m values were observed with increasing substrate concentration, and K_m and k_{cat} values were therefore obtained using both low- and high-substrate-concentration ranges (Table 1). The low-substrate-concentration ranges used to calculate the kinetic parameters were 10 μM to 250 μM (0.11 to 2.8× K_m) for *p*-nitrophenyl-β-D-xylopyranoside, 6 μM to 375 μM (0.08 to 4.7× K_m) for *o*-nitrophenyl-β-D-xylopyranoside, and 104 μM to 2.08 mM (0.03 to 0.63× K_m) for xylobiose. The high-substrate-concentration ranges used were 150 μM to 2,000 μM (0.19 to 2.5× K_m) for *p*-nitrophenyl-β-D-xylopyranoside, 150 μM to 2,000 μM (0.42 to 5.6× K_m) for *o*-nitrophenyl-β-D-xylopyranoside, and 2.08 mM to 16 mM (0.20 to 1.6× K_m) for xylobiose. Kinetic constants were calculated by nonlinear regression fitting to the Michaelis-Menten equation using the program GraFit 5 (6), as shown in Fig. 3A for xylobiose at low substrate concentrations. The nonlinear fit values obtained using either the lower- or higher-substrate-concentration data for the xylopyranosides and xylobiose were similar to those obtained using Lineweaver-Burk plots, as shown for the lower-xylobiose-concentration range used (Fig. 3A, inset). At low substrate concentrations it was found that the K_m values for the β-D-xylopyranosides were both around 0.1 mM, while the K_m for the natural substrate xylobiose was appreciably higher, about 3.3 mM. In contrast, the k_{cat} values obtained under these conditions were similar for the β-D-xylopyranosides and xylobiose, ranging from 2.7 s^{-1} to 4.2 s^{-1} (Table 1). When the kinetic parameters obtained at low substrate concentrations were compared to those measured at high substrate concentrations (Table 1), it was found that the K_m values for *o*-nitrophenyl-β-D-xylopyranoside and xylobiose showed three- to fivefold increases, while the k_{cat} roughly doubled. For *p*-nitrophenyl-β-D-xylopyranoside, the K_m increased about 10-fold and the k_{cat} increased 3-fold, with the result that at high substrate concentrations the k_{cat}/K_m values were the same within the experimental error for both *o*- and *p*-nitrophenyl-β-D-xylopyranosides.

The substrate concentration range used for evaluating xylotriose as a substrate was 52 μM to12 mM (0.36 to 84× K_m). In the course of xylotriose hydrolysis xylobiose is released, which can then be a substrate for hydrolysis. However, the K_m for xylobiose is 3.3 mM, and for the assay using xylotriose the extent of substrate conversion was such that the concentration of the xylobiose released was in the range from 4 to 68 μM. Thus, the effect of subsequent xylobiose hydrolysis on the rate

TABLE 1. Michaelis-Menten parameters for hydrolysis of nitrophenyl-β-D-xylopyranoside and xylooligosaccharide substrates by *Thermoanaerobacterium saccharolyticum* β-xylosidase

Substrate	Concn range (μM)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
p-Nitrophenyl-β-D-xylopyranoside[a]	10–250	0.09 ± 0.01	4.2 ± 0.2	47 ± 6
	150–2,000	0.81 ± 0.19	13 ± 1.4	16 ± 4
o-Nitrophenyl-β-D-xylopyranoside[a]	6–375	0.08 ± 0.01	2.8 ± 0.1	35 ± 5
	150–2,000	0.36 ± 0.06	5.1 ± 0.32	14 ± 2
Xylobiose[a]	104–2,080	3.3 ± 0.7	2.7 ± 0.4	0.82 ± 0.21
	2,080–16,000	10.2 ± 0.7	5.6 ± 0.2	0.55 ± 0.04
Xylotriose[b]	52–12,000	0.143 ± 0.011	2.0 ± 0.1	14 ± 1.2

[a] K_m and k_{cat} were calculated at both lower- and higher-substrate-concentration ranges due to deviation from Michaelis-Menten kinetics at higher substrate concentrations.
[b] Kinetic parameters were obtained from nonlinear fitting to the Michaelis-Menten equation with substrate inhibition (equation 3). The K_i for xylotriose was 1.7 ± 0.1 mM.

measured using xylotriose was minimal. Surprisingly, marked substrate inhibition was observed with xylotriose, necessitating fitting of the data to a substrate uncompetitive inhibition model in which an inactive ES_2 ternary complex is formed (equation 3).

$$v_0 = \frac{k_{cat}[E_0][S]}{K_m + [S] + \left(\frac{[S]^2}{K_i}\right)} \quad (3)$$

Nonlinear curve fitting of the xylotriose hydrolysis data to equation 3 using GraphPad Prism 4 (GraphPad Software, Inc. San Diego, CA) provided estimates for the kinetic parameters, with an R^2 of 0.993 (Fig. 3B). Thus, the K_i for xylotriose was 1.7 ± 0.1 mM, the K_m was 0.143 ± 0.011 mM, the k_{cat} was 2.0 ± 0.1 s^{-1}, and k_{cat}/K_m was 14 ± 1.2 $mM^{-1} \cdot s^{-1}$ (Table 1). It was found that the K_m for xylotriose was about 23-fold lower than that for xylobiose, while the k_{cat} was similar, with the result that the k_{cat}/K_m for xylotriose was 17-fold higher than that for xylobiose measured at lower substrate concentrations.

In the solid-phase variation of the assay utilizing DAB as the peroxidase substrate, an appreciable D-xylose content in the mixed xylooligosaccharide substrate employed resulted in a higher background signal in the agar compared to the results when xylotriose was used as the substrate. However, this did not appear to affect the ability of the assay to detect positive colonies, and the mixed xylooligosaccharide substrate was substantially cheaper than the more chemically pure xylotriose substrate, making it clearly preferable for use in high-throughput screening applications. While clearly visible after 24 h (Fig. 1), the brown precipitate signal was increased after 48 h. Also, development of the signal may have been accelerated by incubating the plates at an elevated temperature. For the imaging step, we found that it was useful to use a flat-bed scanner to image the bottom of the plate with appropriate adjustment of the contrast to minimize any background interference. The image was then flipped about the horizontal axis using standard image-editing software to restore the orientation so that it matched that of the original gridding, and then it was printed and finally scanned using an AlphaImager imaging system (Alpha Innotech Corp., San Leandro, CA).

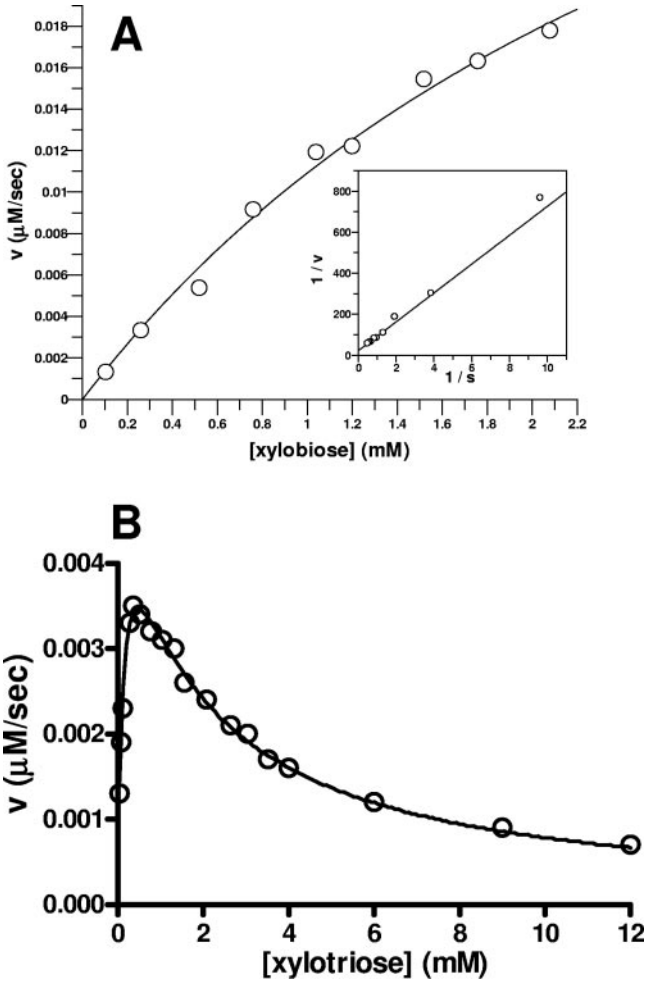
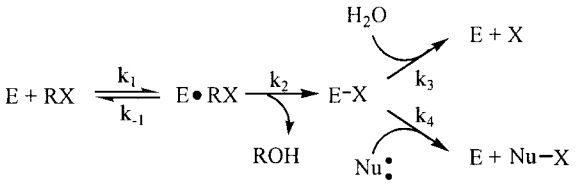


FIG. 3. Reaction conditions were 45°C, 50 mM phosphate (pH 6.0), and 0.1% BSA. (A) Nonlinear regression fitting to the Michaelis-Menten equation of hydrolysis rate versus xylobiose concentration (only 0.104 mM to 2.08 mM shown) using 0.018 μM enzyme. (Inset) Lineweaver-Burk reciprocal plot of the data. (B) Nonlinear regression fitting to the Michaelis-Menten equation with substrate inhibition of hydrolysis rate versus xylotriose concentration using 0.0027 μM enzyme. $R^2 = 0.993$.

DISCUSSION

An enzymatic coupled reaction that quantitatively measures D-xylose has previously been reported for monitoring the consumption of NADH using D-xylose isomerase and D-glucitol dehydrogenase (4). In the xylose quantitation method presented here a coupled enzyme strategy is also used, and this method has the advantage that the enzymes are readily available and hydrogen peroxide is generated as an intermediary analyte, thereby allowing considerable flexibility in the choice of the chromophore or fluorophore used as the final reporter. We found that the xylose assay presented here can be used to obtain useful relative kinetic parameters for a series of natural substrates when it is used to measure the β-xylosidase catalytic activity as described above. Alternatively, the assay can be used to rank a series of β-xylosidases either from naturally occurring enzyme sources or generated in vitro.

Retaining xylosidases have been shown to follow a double-displacement mechanism (5) involving the formation of an enzyme-bound covalent intermediate (xylosylation; k_2), followed by base-catalyzed hydrolysis to form a β-sugar hemiacetal product (dexylosylation; k_3) (equation 4) (2, 16, 21). Alternatively, another substrate molecule or other nucleophile can react with the covalent intermediate E-X in a transglycosylation reaction (k_4).



(4)

By monitoring the hydrolysis of xylobiose by thin-layer chromatography, it has previously been shown that a similar β-xylosidase from a *Thermoanaerobacterium* sp. (94% amino acid identity; Swiss-Prot accession no. P36906) had xylobiose transglycosylation activity (8). Furthermore, detailed kinetic studies by Vocadlo et al. showed that for substrate analogs with pK_as less than 9.0, dexylosylation (k_3) was the rate-limiting step, and deviation from Michaelis-Menten kinetics was observed at higher substrate concentrations due to transglycosylation (16). For the similar β-xylosidase in this study (Swiss-Prot accession no. O30360) we also observed deviation from Michaelian kinetics at higher *o*- and *p*-nitrophenyl-xylopyranoside substrate concentrations (leaving group pK_as, 7.22 and 7.18, respectively), presumably due to transglycosylation. Moreover, transglycosylation was also manifested in the kinetic data for xylobiose hydrolysis, consistent with the conclusion that dexylosylation (k_3) is the rate-limiting step. The rate-limiting nature of the dexylosylation step (k_3) in the xylobiose hydrolysis mechanism is corroborated by the observation (Table 1) that the k_{cat} values are similar for the nitrophenyl-β-D-xylopyranoside substrates and the natural substrates, in spite of the large difference in the pK_as of the leaving groups (pK_a of xylose, ~12.2). It has been pointed out that natural substrates can have k_{cat} values as good as artificial substrates with better leaving groups, owing to (evolutionary) optimization of the stability of the transition state associated with their hydrolysis, as discussed briefly in the review of Zechel and Withers (21).

Interestingly, significant substrate inhibition was observed using the natural substrate xylotriose. Substrate inhibition has been observed previously for a *Bacillus licheniformis* 1,3-1,4-β-glucanase (11) and a *Streptomyces* sp. strain QM-B814 β-glucosidase (Bgl3) (15). At low substrate concentrations the β-xylosidase enzyme appears to be a more specific catalyst for hydrolysis of xylotriose than for hydrolysis of xylobiose, with a 17-fold-higher k_{cat}/K_m (Table 1), due mainly to a much lower K_m.

The solid-phase assay described above has the potential to effectively screen genomic and in vitro mutagenized enzyme libraries, which should have applications in gene discovery and directed-evolution studies. This assay is particularly useful since previously described solid-phase β-xylosidase assays involve detection of the release of a chromophore or fluorophore moiety from the corresponding xylopyranoside substrate. The fluorophores and chromophores employed tend to diffuse rapidly in the agar, making detection of bacterial colonies carrying the gene of interest problematic. Also, the use here of natural xylooligosaccharide substrates rather than artificial substrates results in increased selectivity for detection of bona fide hemicellulase activity. Additionally, we demonstrated (data not shown) that when the expression host is gridded in either a 96- or 384-well format, the membrane can be imaged and the pixel density can be readily quantitated using an AlphaImager imaging system (Alpha Innotech Corp., San Leandro, CA). This is especially useful in screening libraries of enzyme variants since a list of candidates can be generated for automated picking in a subsequent rearraying step. Moreover, it may be possible to add further screening criteria to the solid-phase assay to allow simultaneous screening and selection for desired properties, such as protein expression, substrate specificity, thermal stability, and/or pH stability.

Recently, the regiospecificity of deacetylation of a series of monoacetylated 4-nitrophenyl-β-D-xylopyranoside substrate analogs by a deacetylase activity has been evaluated by using β-xylosidase enzyme activity as a reporter (1). The β-xylosidase assay described here in which natural substrates are used should extend this use of β-xylosidase as an auxiliary reporting enzyme to quantitation of the xylose generated from natural hemicellulose substrates by various mixtures of wild-type or mutant enzymes or, alternatively, the xylose incorporation rate in the case of biosynthetic pathways. Thus, we anticipate that the extent of breakdown of xylan by a commercial xylanase preparation can be measured with the assay described here, using the resulting soluble xylooligosaccharide mixture as the substrate source and monitoring the xylose monosaccharide released. This could be useful as a process development strategy that directs the in vitro evolution of multienzyme modules of biodegradation or biosynthetic pathways by measuring the concentration of only the final or initial metabolite whose rate of formation or incorporation is to be optimized. Also, the hydrolyzability of various xylooligosaccharide substrate mixtures can be assessed, which is important in estimating the ability of a given β-xylosidase to efficiently hydrolyze potential xylooligosaccharide mixtures generated by upstream hemicellulolytic steps.

In conclusion, here we describe a sensitive enzyme-coupled

assay for xylose in which readily available enzymes that can be coupled to a large selection of chromophores or fluorophores are used. We have also demonstrated the utility of this assay in β-xylosidase solution and solid-phase assays using natural xylooligosaccharide substrates. Work is in progress to employ these assays in a high-throughput format to screen β-xylosidase enzyme libraries for mutants with improved stability and kinetic properties for use in the enzymatic breakdown of hemicellulose.

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